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Phospholipase Cβ Serves as a Coincidence Detector through Its Ca^{2+} Dependency for Triggering Retrograde Endocannabinoid Signal

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Summary

Endocannabinoids mediate retrograde signal and modulate transmission efficacy at various central synapses. Although endocannabinoid release is induced by either depolarization or activation of $G_{q/11}$-coupled receptors, it is markedly enhanced by the coincidence of depolarization and receptor activation. Here we report that this coincidence is detected by phospholipase Cβ1 (PLCβ1) in hippocampal neurons. By measuring cannabinoid-sensitive synaptic currents, we found that the receptor-driven endocannabinoid release was dependent on physiological levels of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), and markedly enhanced by depolarization-induced $[Ca^{2+}]_i$ elevation. Furthermore, we measured PLC activity in intact neurons by using exogenous TRPC6 channel as a biosensor for the PLC product diacylglycerol and found that the receptor-driven PLC activation exhibited similar $[Ca^{2+}]_i$ dependence to that of endocannabinoid release. Neither endocannabinoid release nor PLC activation was induced by receptor activation in PLCβ1 knockout mice. We therefore conclude that PLCβ1 serves as a coincidence detector through its Ca^{2+} dependency for endocannabinoid release in hippocampal neurons.

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Introduction

Coincidence detection of two distinct signaling events is important for various aspects of neuronal function (Konnerth et al., 1996). The most well-known example is the NMDA receptor, which requires both glutamate binding and membrane depolarization for its channel opening and thereby can detect the coincidence of presynaptic activity (i.e., glutamate release) and postsynaptic depolarization (Cotman et al., 1988). This mechanism is prerequisite for the induction of long-term potentiation in various synapses (Bliss and Collingridge, 1993; Malenka and Nicoll, 1999). Another example is the inositol-1,4,5-trisphosphate receptor (IP_3R) that requires both IP_3 and Ca^{2+} for its activation to induce Ca^{2+} release from internal stores (Bezprozvanny et al., 1991; Finch et al., 1991; Iino, 1990). IP_3R can therefore detect the coincidence of receptor activation (i.e., IP_3 production) and postsynaptic activity (i.e., spike-mediated Ca^{2+} entry) and is thought to contribute to supralinear Ca^{2+} signals (Berridge, 1998; Nakamura et al., 1999; Simpson et al., 1995; Wang et al., 2000).

A mechanism of coincidence detection may also play an important role in regulating retrograde endocannabinoid signaling, which contributes to short-term and long-term synaptic plasticity in various brain regions, including hippocampus, cerebellum, neocortex, amygdala, and basal ganglia (Alger, 2002; Chevaleyre and Castillo, 2003; Gerdeman et al., 2002; Kano et al., 2002; Kreitzer and Regehr, 2002; Marsicano et al., 2002; Sjostrom et al., 2003; Wilson and Nicoll, 2002). Endocannabinoids are released from postsynaptic neurons and suppress the transmitter release retrogradely by activating presynaptic cannabinoid receptor type 1 (CB1R) (Kreitzer and Regehr, 2002; Maejima et al., 2001b; Wilson and Nicoll, 2002). The endocannabinoid release is triggered by either depolarization (Diana et al., 2002; Kreitzer and Regehr, 2001; Ohno-Shosaku et al., 2001, 2002b; Wilson and Nicoll, 2001; Yoshida et al., 2002) or activation of $G_{q/11}$-coupled receptors such as group I metabotropic glutamate receptors (I-mGluRs) (Maejima et al., 2001a; Ohno-Shosaku et al., 2002a; Varma et al., 2001) and M_1/M_3 muscarinic receptors (Fukudome et al., 2004). Importantly, the endocannabinoid release is markedly facilitated when depolarization is combined with the activation of these receptors (Kim et al., 2002; Ohno-Shosaku et al., 2002a, 2003). Therefore, some mechanism must exist to integrate two separate signals, namely, depolarization and receptor activation, for induction of endocannabinoid release.

Two major endocannabinoids that have been identified are anandamide (Devane et al., 1992) and 2-arachidonoylglycerol (2-AG) (Mechoulam et al., 1995; Sugiura et al., 1995). Anandamide is biosynthesized by *N*-acyltransferase and phospholipase D, whereas 2-AG is produced by phospholipase C (PLC) and diacylglycerol (DAG) lipase (Di Marzo et al., 1998; Piomelli et al., 2000; Stella et al., 1997). Among five types (β, γ, δ, ϵ, and ζ) of PLC, PLCβ isozymes are activated by $G_{q/11}$-coupled receptors including I-mGluRs and M_1/M_3 muscarinic re-

ceptors (Rebecchi and Pentyala, 2000; Rhee, 2001). It is also known that all five types of PLC require Ca^{2+} for catalytic function, although the Ca^{2+} concentration needed for maximum activation is different in different PLC isozymes (Allen et al., 1997; Kelley et al., 2001; Kouchi et al., 2004; Rebecchi and Pentyala, 2000; Taylor et al., 1991). Thus, if PLCβ activation is required for endocannabinoid release and is sensitive to $[Ca^{2+}]_i$ elevation in a physiological range, PLCβ can serve as a coincidence detector for endocannabinoid release.

In the present study, we examined the role of PLCβ in endocannabinoid release and analyzed the $[Ca^{2+}]_i$ dependence of PLCβ activation in intact hippocampal neurons. We report that the receptor-driven endocannabinoid release requires PLCβ1, the one of the four PLCβ isozymes enriched in the hippocampus, and depends sharply on $[Ca^{2+}]_i$ in a physiological range. We also demonstrate that the receptor-driven PLCβ1 activation exhibits a similar $[Ca^{2+}]_i$ dependence to that of the endocannabinoid release. We therefore conclude that PLCβ1 serves as a coincidence detector through its Ca^{2+} dependency for triggering retrograde endocannabinoid signal in hippocampal neurons.

Results

Synaptic Activation of Muscarinic Receptors Enhances DSI

It is well known that postsynaptic depolarization triggers the release of endocannabinoids that retrogradely suppress inhibitory postsynaptic currents (IPSCs), a phenomenon termed "depolarization-induced suppression of inhibition" (DSI) (Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001). It is also known that the activation of either M_1/M_3 muscarinic receptors or group I mGluRs markedly enhances DSI by facilitating depolarization-induced endocannabinoid release, in slices as well as culture preparations (Kim et al., 2002; Ohno-Shosaku et al., 2002a, 2003; Varma et al., 2001). We first examined whether synaptically released acetylcholine (Cole and Nicoll, 1984) can enhance DSI in mouse hippocampal slices (Figure 1). Evoked IPSCs were recorded from CA1 pyramidal neurons, and DSI was induced by depolarization (0 mV, 3 s). The stratum oriens stimulation (100 Hz, 1 s) did not influence IPSCs by itself, but markedly enhanced DSI when it was applied during depolarization (Figures 1A and 1B). This enhancement was significantly reduced by atropine (1 μM), confirming the contribution of muscarinic acetylcholine receptors to the DSI enhancement. Then we examined the timing dependence, by changing the relative timing of depolarization and oriens stimulation (Figure 1C). In this set of experiments, we started oriens stimulation (100 Hz, 0.2 s) at 0 s (simultaneously), 1 s, 2 s, or 3 s after the onset of depolarization (0 mV, 3 s). The enhancement of DSI was most effective when oriens stimulation was applied during the latter half of depolarization. When oriens stimulation was applied just after the end of depolarization, no enhancement was observed (Figure 1C, $t_{OR\text{-}stim.} - t_{Depol.} = 3$). In the following experiments, we used cultured hippocampal neurons to identify the molecule(s) that can detect the coincidence of depolarization and receptor activation for triggering endocannabinoid release.

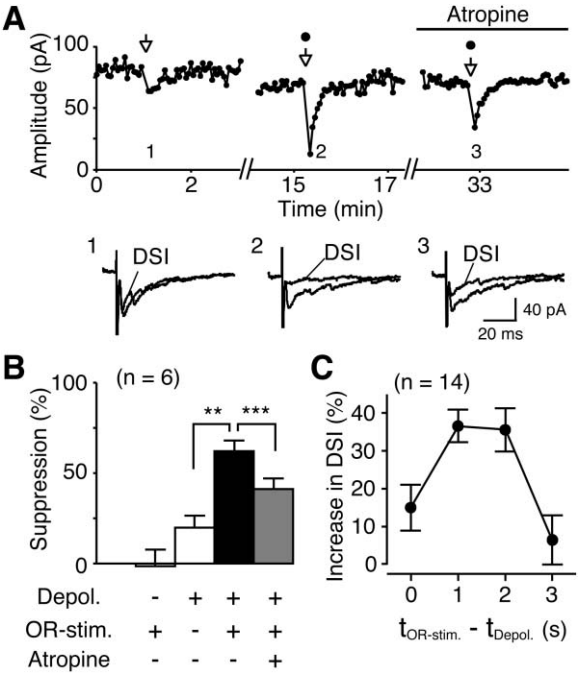

Figure 1. Synaptic Activation of Muscarinic Receptors Enhances DSI in Mouse Hippocampal Slices

(A) An example showing enhancement of DSI by synaptic activation of cholinergic inputs and its inhibition by atropine. IPSCs were evoked at 0.33 Hz by the stimulation of the stratum radiatum. Oriens stimulation (100 Hz, 1 s; filled circles) was applied during the latter half of depolarization (0 mV, 3 s; downward arrows). IPSC traces acquired at the indicated time points (1–3) are shown on the lower panel. Each trace is the average of three consecutive IPSCs acquired before or after depolarization.

(B) Averaged data for the suppression of IPSC amplitude by 3 s depolarization alone, by the oriens stimulation (100 Hz, 1 s) alone, and by the depolarization plus oriens stimulation in the presence or absence of 1 μM atropine.

(C) Averaged data for the timing dependence of DSI enhancement. Ordinate, increase in DSI magnitude; abscissa, time at which oriens stimulation (100 Hz, 0.2 s) was started after the onset of depolarization (0 mV, 3 s).

Involvement of PLCβ1 in Agonist-Induced Endocannabinoid Release

Endocannabinoid release can be induced either by depolarization or by activation of I-mGluRs (Maejima et al., 2001a) or M_1/M_3 muscarinic receptors (Fukudome et al., 2004), which are coupled to PLCβ. Among four isoforms of PLCβ, PLCβ1 is the major isoform in the hippocampus (Watanabe et al., 1998). We therefore examined whether PLCβ1 was involved in endocannabinoid release. We performed paired whole-cell recordings from cultured hippocampal neurons prepared from wild-type or PLCβ1 knockout mice and recorded IPSCs from one of the pair (the postsynaptic neuron) evoked by stimulating the other (the presynaptic neuron). IPSCs in hippocampal neurons can be classified as cannabinoid-sensitive and cannabinoid-insensitive types (Ohno-Shosaku et al., 2001). In the following experiments, only cannabinoid-sensitive IPSCs were analyzed. The cannabinoid sensitivity of IPSCs was determined by applying the endocannabinoid 2-AG (Figures 2A and 2B).

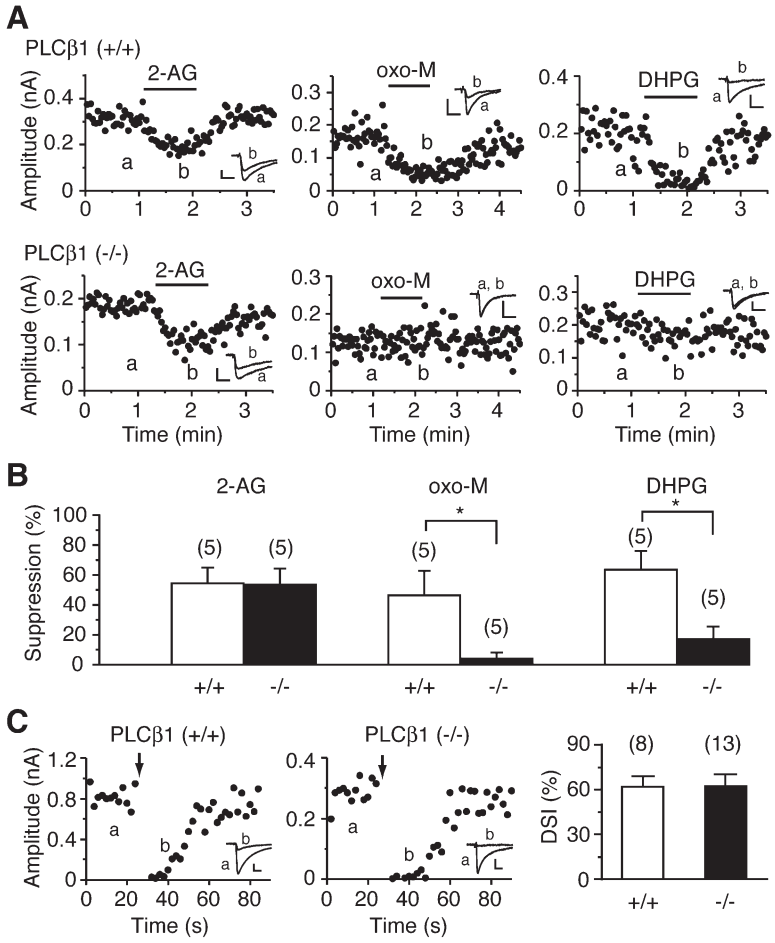

Figure 2. Lack of Agonist-Induced Endocannabinoid Release in PLCβ1 Knockout Mice

Cannabinoid-sensitive IPSCs were recorded in neurons prepared from wild-type [PLCβ1 (+/+)] or PLCβ1 knockout [PLCβ1 (−/−)] mice. Neurons were dialyzed with a pCa 6 solution containing 10 mM EGTA (A and B) or a 5 mM EGTA solution containing no added Ca^{2+} (C). (A) Representative data showing effects of 2-AG (0.1 μM), oxo-M (0.3 μM), and DHPG (5 μM) on IPSCs in wild-type and PLCβ1 knockout neurons. The amplitude of IPSC is plotted as a function of time. (Inset) IPSC traces acquired at the indicated time points. In this and the following figures, each trace is the average of 6 to 15 consecutive IPSCs. Calibration bars, 0.1 nA, 20 ms. (B) Averaged data for percent suppression of IPSC amplitudes by 2-AG, oxo-M, and DHPG in wild-type and PLCβ1 knockout neurons. (C) Depolarization-induced endocannabinoid release was normal in PLCβ1 knockout neurons. Example (left) and averaged data (right) showing depolarization-induced suppression of IPSC in wild-type and PLCβ1 knockout neurons. Postsynaptic neurons were depolarized from −80 to 0 mV for 5 s at the time points indicated by arrows. Two IPSC traces obtained before and after depolarization are shown. Calibration bars, 0.2 nA, 10 ms for wild-type; 0.1 nA, 10 ms for PLCβ1 knockout neurons. Numbers of tested cells are indicated in parentheses for this and subsequent figures.

Application of the muscarinic agonist oxotremorine-M (oxo-M, 0.3 μM) or the I-mGluR agonist (RS)-3,5-dihydroxyphenylglycine (DHPG, 5 μM) induced a suppression of IPSC in neurons from wild-type mice (Figures 2A and 2B). Previous studies have revealed that the suppression induced by these agonists is caused by endocannabinoids that are released from postsynaptic neurons (Fukudome et al., 2004; Ohno-Shosaku et al., 2002a). In PLCβ1 knockout neurons, neither oxo-M nor DHPG caused clear suppression of IPSC (Figures 2A and 2B). The lack of suppression should be attributable to the defect of endocannabinoid release, because the presynaptic cannabinoid sensitivity was normal in these neurons (Figures 2A and 2B). Thus, these results indicate that PLCβ1 activity is required for receptor-driven endocannabinoid release.

We then examined whether PLCβ1 was required for depolarization-induced endocannabinoid release. Postsynaptic depolarization (to 0 mV, 5 s) induced a transient suppression of IPSC (DSI) in wild-type neurons. It is generally accepted that DSI is mediated by endocannabinoids released from postsynaptic neurons in response to depolarization-induced $[Ca^{2+}]_i$ elevation (Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001). In contrast to the agonist-induced suppression, DSI was intact in PLCβ1 knockout neurons (Figure 2C), indicating clearly that PLCβ1 is not required for the endocannabinoid release induced by depolarization alone.

$[Ca^{2+}]_i$ Dependence of Agonist-Induced Endocannabinoid Release

We next investigated the $[Ca^{2+}]_i$ dependence of agonist-induced endocannabinoid release in rat hippocampal neurons. Postsynaptic neurons were dialyzed with various pCa solutions containing 10–30 mM BAPTA through patch pipettes. Possible changes in local $[Ca^{2+}]_i$ caused by IP_3-induced Ca^{2+} release should be blocked under these conditions (Nakamura et al., 2000). Cannabinoid-sensitive IPSCs were selected by applying 2-AG (30 nM), and then the effects of oxo-M (0.3 μM) on IPSCs were examined. As exemplified in Figure 3A, oxo-M-induced suppression was evident at pCa 6, but was not detectable at pCa 8 or pCa 9. The $[Ca^{2+}]_i$ dependence of oxo-M-induced suppression is summarized in Figure 3B. It should be noted that essentially the same $[Ca^{2+}]_i$ dependence was obtained with the internal solutions containing 10 mM EGTA instead of BAPTA (Figure 3B). Dialyzing postsynaptic neurons with various pCa solutions did not influence presynaptic cannabinoid sensitivity (Figure 3C). Thus, the $[Ca^{2+}]_i$ dependence of oxo-M-induced suppression directly reflects that of endocannabinoid release from postsynaptic neurons. Thus,

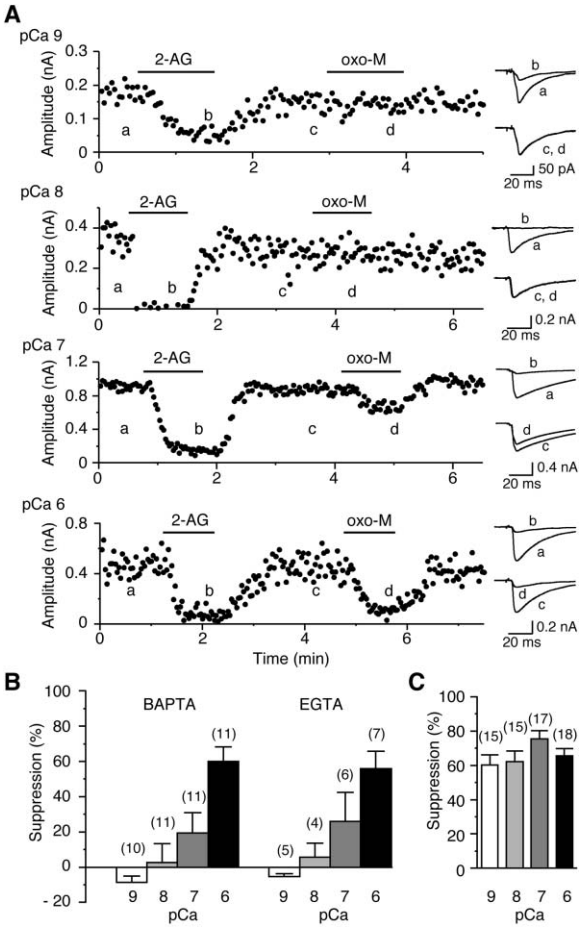

Figure 3. $[Ca^{2+}]_i$ Dependence of oxo-M-Induced Endocannabinoid Release

Cannabinoid-sensitive IPSCs were recorded from rat neurons dialyzed with the indicated pCa solutions. (A) Examples representing the effects of 2-AG (30 nM) and oxo-M (0.3 μM) at four different pCa levels buffered with 30 mM BAPTA. IPSC traces acquired at the indicated time points are shown on the right. (B) Averaged data for oxo-M-induced suppression of IPSC at four different pCa levels buffered with 10–30 mM BAPTA (BAPTA) or 10 mM EGTA (EGTA). (C) Averaged data for 2-AG-induced suppression at four different pCa levels.

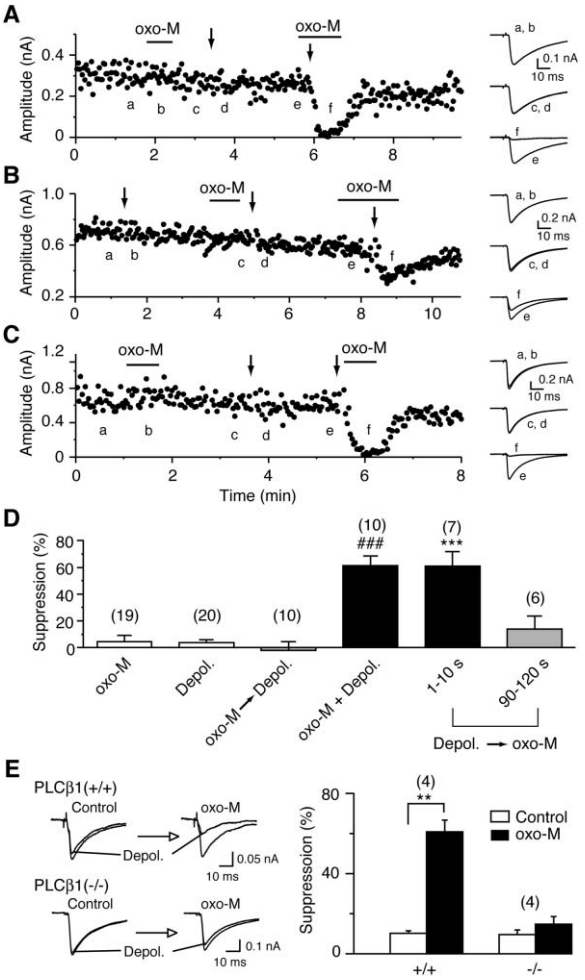

Figure 4. Endocannabinoid Release Induced by oxo-M Application Combined with Depolarization

Cannabinoid-sensitive IPSCs were recorded from rat neurons dialyzed with a pCa 8 solution buffered with 10 mM BAPTA (A–D) or mouse neurons dialyzed with a 5 mM EGTA solution (E). (A–C) Examples representing the effects of oxo-M (0.3 μM) application combined with postsynaptic depolarization (arrows) on IPSC at various intervals. (D) Averaged data for suppression induced by oxo-M application alone (oxo-M), depolarization alone (Depol.), depolarization 30 s after oxo-M-treatment (oxo-M→Depol.), and oxo-M application 1–10 s or 90–120 s after depolarization (Depol.→oxo-M). Asterisks and sharps indicate significant differences compared to the suppression induced by oxo-M application alone and by depolarization alone, respectively. (E) Sample traces (left) and averaged data (right) for depolarization-induced suppression of IPSC in the absence (control) or presence of 0.3 μM oxo-M in neurons prepared from wild-type [PLCβ1 (+/+)] or PLCβ1 knockout [PLCβ1 (−/−)] mice. In this series of experiments, we first applied depolarizing pulses with increasing durations (0.1, 0.2, 0.5, 1, 2, 3, 4, and 5 s) until DSI (more than 15% suppression) was induced. The maximal pulse duration just subthreshold for DSI was used for the rest of the experiment.

these data indicate that the oxo-M-induced endocannabinoid release that requires PLCβ1 is strongly dependent on $[Ca^{2+}]_i$ in a physiological range.

Enhancement of Endocannabinoid Release by Transient $[Ca^{2+}]_i$ Elevation

Next, we examined the effects of depolarization-induced transient elevation of $[Ca^{2+}]_i$ on agonist-induced endocannabinoid release using rat neurons. In these experiments, postsynaptic neurons were dialyzed with a pCa 8 solution containing 10 mM BAPTA. To minimize the possible contribution of PLCβ1-independent endocannabinoid release induced by depolarization alone (DSI, see Figure 2C), we adjusted the duration of depolarization in each experiment so as not to induce DSI. When application of 0.3 μM oxo-M and depolarization were temporally separate, suppression of IPSC was not induced (Figure 4A). In contrast, when depolarization was applied during oxo-M application, marked suppression of IPSC was induced (Figure 4A, the second arrow). Similar enhancement of suppression has been reported when depolarization was applied during agonist application (Kim et al., 2002; Ohno-Shosaku et al., 2002a, 2003;

Varma et al., 2001). In these previous studies, it is clearly shown that the enhanced suppression is caused by the enhanced endocannabinoid release (Ohno-Shosaku et al., 2002a, 2003). In the present study, we further examined the effective timing of agonist application and depolarization for the enhancement. When oxo-M application was followed by depolarization, IPSCs were not affected (Figure 4B, the second arrow; Figure 4D, oxo-M→Depol.). In marked contrast, when depolarization was immediately followed by oxo-M application, IPSCs were significantly suppressed (Figure 4C, the second bar; Figure 4D, Depol→oxo-M, 1–10 s). Under this condition (10 mM BAPTA), $[Ca^{2+}]_i$ was considerably elevated at the time of oxo-M application (see Figure 8D). These data clearly indicate that endocannabinoids are effectively released when oxo-M application coincides with $[Ca^{2+}]_i$ elevation.

We confirmed that this enhancement of endocannabinoid release requires PLCβ1, using PLCβ1 knockout mice. When weak depolarization, which did not induce DSI by itself, was combined with oxo-M application, a marked suppression of IPSC was observed in wild-type neurons (Figure 4E). By contrast, the same treatment failed to induce clear suppression in neurons prepared from PLCβ1 knockout mice (Figure 4E). Taken together, these results strongly indicate that the PLCβ1-dependent endocannabinoid release is $[Ca^{2+}]_i$ dependent and can be markedly enhanced by depolarization-induced $[Ca^{2+}]_i$ elevation.

Real-Time Measurements of PLCβ Activity by Recording TRPC6 Currents

To examine the $[Ca^{2+}]_i$ dependence of PLCβ1 activity, we used a newly developed electrophysiological method with TRPC6 channel, a member of the canonical transient receptor potential family (Minke and Cook, 2002). This channel is activated by the PLC product DAG (Trebak et al., 2003) and therefore can be used as a biosensor for PLC activity (Delmas et al., 2002). We first confirmed that the exogenously expressed TRPC6 channel can be used as a DAG sensor in rat cultured hippocampal neurons. Application of a membrane-permeable DAG analog, 1-oleoyl-2-acetyl-sn-glycerol (OAG, 50 μM), induced a large inward current in the neurons expressing exogenous TRPC6 channels, but not in control neurons (Figures 5A and 5C). When PLCβ-coupled receptors were activated by applying either 3 μM oxo-M or 50 μM DHPG, large inward currents were induced in TRPC6-expressing neurons (Figures 5B and 5C). These agonist-induced currents were negligible in control neurons, indicating that exogenous TRPC6 channels mediate the currents. The pretreatment of TRPC6-expressing neurons with the PLC inhibitor U73122 (5 μM) for >10 min did not affect the OAG-induced current (Figure 5C), but suppressed the agonist-induced currents completely (Figures 5B and 5C). Application of thapsigargin (0.5–1 μM), a drug inducing store depletion, induced no detectable currents by itself ($n = 4$, data not shown), and barely affected the oxo-M-induced current (84.5% ± 16.2% of control; $p > 0.5$; $n = 19$). All these results are consistent with previous studies showing that TRPC6 is a DAG-activated channel (Delmas et al., 2002; Hofmann et al., 1999; Inoue et al., 2001; Zhang and Saffen, 2001) and show that the agonist-induced PLCβ activation can be monitored by measuring TRPC6 currents as an indicator of DAG production.

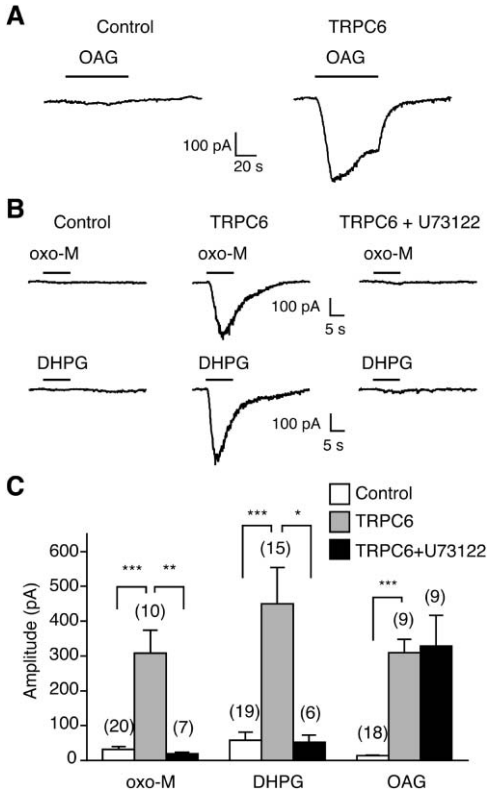

Figure 5. Activation of Exogenous TRPC6 Channels by OAG, oxo-M, and DHPG

Rat cultured hippocampal neurons were dialyzed with a pCa 7 solution containing 10 mM BAPTA. (A) Sample traces showing the effects of OAG (50 μM) in control and TRPC6-expressing neurons. (B) Sample traces showing effects of oxo-M (3 μM) and DHPG (50 μM). In control neurons, these agonists induced no detectable responses. In TRPC6-expressing neurons, the agonists induced marked inward currents. These responses were suppressed by the treatment with the PLC inhibitor U73122 (5 μM). (C) Averaged amplitudes of inward currents induced by oxo-M, DHPG, and OAG in control neurons and TRPC6-expressing neurons with or without U73122 treatment.

PLCβ1 Is Required for Agonist-Induced TRPC6 Activation

We then examined the contribution of PLCβ1 to these agonist-induced TRPC6 currents, using PLCβ1 knockout mice. When oxo-M and DHPG were applied to TRPC6-expressing neurons prepared from wild-type (*PLCβ1*$^{+/+}$) or knockout (*PLCβ1*$^{-/-}$) mice, large inward currents were induced in wild-type neurons, but not in PLCβ1 knockout neurons (Figure 6). These results clearly demonstrate that PLCβ1 is the functionally major PLCβ isoform in hippocampal neurons and that TRPC6 currents induced by oxo-M or DHPG reflect the PLCβ1 activity in our experimental conditions.

PLCβ1 Activation Is Dependent on $[Ca^{2+}]_i$

We next examined the $[Ca^{2+}]_i$ dependence of PLCβ1 activation in native rat hippocampal neurons expressing

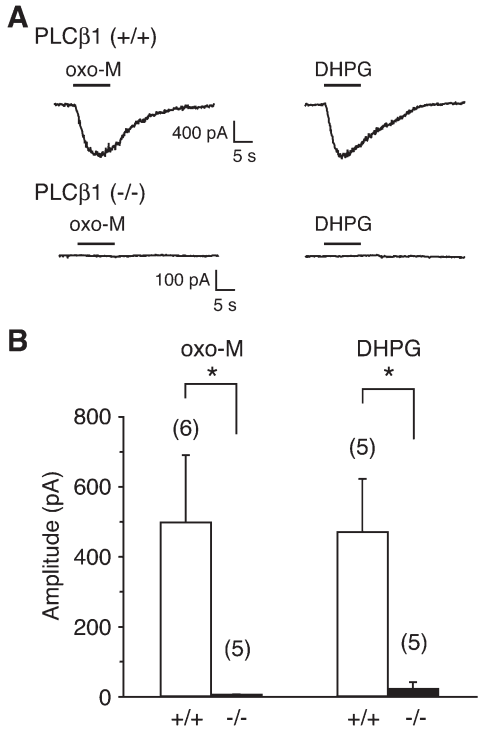

Figure 6. Lack of Agonist-Induced TRPC6 Currents in PLCβ1 Knockout Mice

TRPC6-expressing neurons from wild-type [PLCβ1 (+/+)] or PLCβ1 knockout [PLCβ1 (−/−)] mice were dialyzed with a pCa 7 solution containing 10 mM BAPTA. (A) Sample traces showing the effects of oxo-M (3 μM) and DHPG (50 μM). These agonists induced large inward currents in wild-type neurons, but not in PLCβ1 knockout neurons. (B) Averaged amplitudes of inward currents induced by the two agonists in wild-type and PLCβ1 knockout neurons.

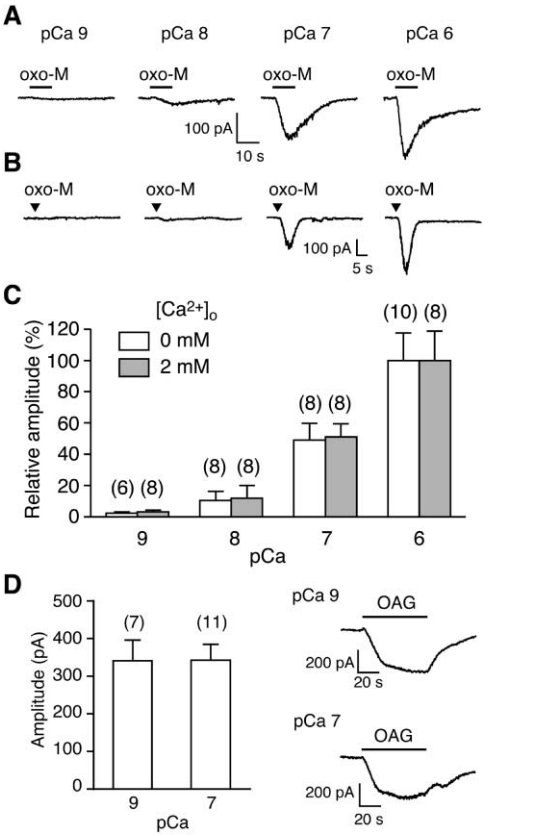

Figure 7. $[Ca^{2+}]_i$ Dependence of Receptor-Driven PLCβ1 Activation

Rat neurons expressing TRPC6 were dialyzed with 10 mM BAPTA-containing solutions with the indicated pCa. (A–C) Sample traces (A and B) and averaged amplitudes (C) of oxo-M (3 μM)-induced currents at four different pCa levels. The external solution contained 0 mM (A) or 2 mM Ca^{2+} (B). Oxo-M was applied with a 1 ms command pulse in (B) to minimize Ca^{2+} influx through TRPC6 channels. In the summary bar graphs (C), the amplitudes were normalized to the values for pCa 6. (D) Averaged amplitudes (left) and sample traces (right) of OAG-induced currents in the absence of external Ca^{2+} with a pCa 9 or pCa 7 internal solution.

exogenous TRPC6. Neurons were dialyzed with pipette solutions containing 10 mM BAPTA and various concentrations of Ca^{2+}. To exclude possible disturbance of local $[Ca^{2+}]_i$ by Ca^{2+} influx through TRPC6 channels, we applied oxo-M in the absence of external Ca^{2+}. As shown in Figure 7A, the amplitude of oxo-M-induced TRPC6 current was sharply dependent on the free Ca^{2+} concentration of the pipette solutions. This Ca^{2+} dependence of TRPC6 current was not derived from the Ca^{2+} dependence of TRPC6 channel itself, because direct activation of TRPC6 channel by OAG was not influenced by $[Ca^{2+}]_i$ (Figure 7D). We also examined possible influence of calmodulin, since it is reported that TRPC6 channel is modulated by Ca^{2+}-calmodulin (Boulay, 2002). However, the oxo-M-induced current was not influenced by the calmodulin inhibitor calmidazolium (2 μM for >10 min, 92.6% ± 8.1% of control, n = 6), which is consistent with the study using sympathetic neurons (Delmas et al., 2002). These results clearly indicate that agonist-induced PLCβ1 activation itself is Ca^{2+} dependent.

Similar experiments were performed in the presence of a physiological level of external Ca^{2+} (2 mM). In this set of experiments, we decreased the duration of oxo-M application (1 ms opening of local perfusion valve, see Experimental Procedures) in order to minimize Ca^{2+} influx through TRPC6 channels and thus possible local $[Ca^{2+}]_i$ elevation. The Ca^{2+} dependence of oxo-M-induced currents in this condition (Figures 7B and 7C, filled columns) was very similar to that obtained in the absence of external Ca^{2+} (Figure 7C, open columns). These data indicate that the agonist-induced PLCβ1 activation is sharply dependent on $[Ca^{2+}]_i$ around the resting level (less than 100 nM), irrespective of the level of external Ca^{2+}. Since the $[Ca^{2+}]_i$ dependence of PLCβ1 activation (Figure 7C) is essentially the same as that of endocannabinoid release (Figure 3), it is strongly suggested that PLCβ1 activation is a rate-limiting step of agonist-induced endocannabinoid release.

Enhancement of Agonist-Induced PLCβ1 Activation by Transient $[Ca^{2+}]_i$ Elevation

Finally, we examined whether the agonist-induced PLCβ1 activation is influenced by depolarization-induced transient elevation of $[Ca^{2+}]_i$. In rat TRPC6-expressing neurons dialyzed with a pCa 8 solution, oxo-M (3 μM) was briefly applied three times with an interval of 90 s. The neurons were depolarized from −80

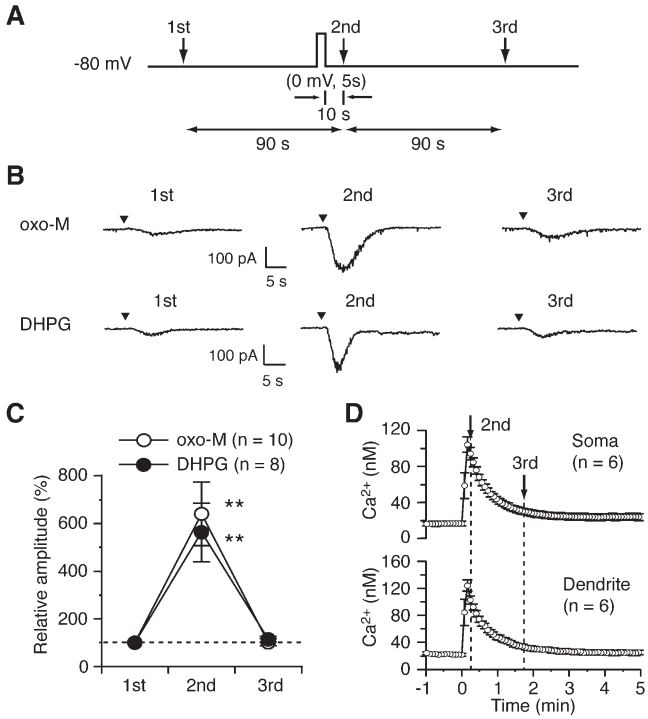

Figure 8. Enhancement of Receptor-Driven PLCβ1 Activation by Depolarization-Induced $[Ca^{2+}]_i$ Elevation

Rat TRPC6-expressing neurons were dialyzed with a pCa 8 solution containing 10 mM BAPTA. The external solution contained 2 mM Ca^{2+}. (A) Schematic diagram for experimental protocol. An agonist was applied three times (downward arrows, with 1 ms pulse), and neurons were depolarized 10 s before the second agonist application. (B) Sample traces of inward currents induced by brief application (arrow heads) of oxo-M (3 μM) or DHPG (50 μM). (C) Averaged data for enhancement of oxo-M- or DHPG-induced currents by preceding depolarization. The amplitudes were normalized to the values for the first agonist application. (D) Averaged time courses of $[Ca^{2+}]_i$ change induced by 5 s depolarization at the soma and dendrites (n = 6). Downward arrows indicate the time points for the second and the third agonist applications.

to 0 mV for 5 s just before the second application (Figure 8A). The oxo-M-induced current was markedly enhanced at the second application and returned to the original level at the third application (Figures 8B and 8C). Figure 8D presents the averaged time courses of depolarization-induced $[Ca^{2+}]_i$ elevation at the soma and dendrites. The $[Ca^{2+}]_i$ was significantly elevated at the time of the second application, but decreased near the original level at the time of the third application (Figure 8D). The DHPG-induced current was also enhanced by the preceding depolarization (Figures 8B and 8C). These data indicate that the agonist-induced PLCβ1 activation is greatly enhanced when it coincides with depolarization-induced $[Ca^{2+}]_i$ elevation. Taken together, the results in the present study clearly indicate that PLCβ1 can detect the coincidence of receptor activation and depolarization ($[Ca^{2+}]_i$ elevation) and trigger endocannabinoid release in hippocampal neurons.

Discussion

In the present study, we observed that a marked IPSC suppression is induced in hippocampal slices when depolarization and synaptic activation of muscarinic receptors coincide. To elucidate mechanisms underlying this coincidence detection, we used cultured hippocampal neurons and determined whether PLCβ can serve as a coincidence detector for triggering endocannabinoid release. We found that the receptor-driven endocannabinoid release requires PLCβ1. We also found that this PLCβ1-mediated endocannabinoid release was strongly dependent on the basal levels of $[Ca^{2+}]_i$, and augmented by depolarization-induced transient elevation of $[Ca^{2+}]_i$. We next examined the $[Ca^{2+}]_i$ dependence of PLCβ1 activity in intact neurons, using exogenously expressed TRPC6 channel as a biosensor for the PLC product DAG. We found that the receptor-driven PLCβ1 activation exhibits a similar $[Ca^{2+}]_i$ dependence to that of receptor-driven endocannabinoid release and can be markedly augmented by depolarization-induced $[Ca^{2+}]_i$ elevation. From these results, we conclude that PLCβ1 is a key element in endocannabinoid release and that it can serve as a coincidence detector through its Ca^{2+} dependency for triggering retrograde endocannabinoid signal in hippocampal neurons.

The Molecular Identity of Endocannabinoids

Recent studies have revealed that endocannabinoids act as retrograde messengers and contribute to short-term and long-term synaptic plasticity at various brain regions (Alger, 2002; Chevaleyre and Castillo, 2003; Gerdeman et al., 2002; Kano et al., 2002; Kreitzer and Regehr, 2002; Marsicano et al., 2002; Sjostrom et al., 2003; Wilson and Nicoll, 2002). Endocannabinoids are produced and released from postsynaptic neurons in response to either depolarization or activation of I-mGluRs or M_1/M_3 receptors and then suppress the transmitter release through activating presynaptic CB1 receptors (Alger, 2002; Kano et al., 2002; Kreitzer and Regehr, 2002; Maejima et al., 2001b; Wilson and Nicoll, 2002). The molecular identity of the endocannabinoids mediating this retrograde suppression, however, has not been established.

Two major endocannabinoids, anandamide and 2-AG, are produced through different biosynthesis pathways. Anandamide is produced by *N*-acyltransferase and phospholipase D, whereas 2-AG is produced by PLC and DAG lipase (Di Marzo et al., 1998; Piomelli et al., 2000; Stella et al., 1997). By examining effects of the PLC inhibitor U73122 and a DAG lipase inhibitor (RHC-

80267), it has been suggested that 2-AG mediates I-mGluR-dependent retrograde suppression in the hippocampus (Chevaleyre and Castillo, 2003). In our culture system, however, unfavorable side effects were observed with these drugs. The treatment with U73122 affected the presynaptic cannabinoid sensitivity, and application of RHC-80267 induced a severe rundown of IPSCs. Thus, instead of using these drugs, we attempted an alternative approach in the present study. We demonstrate clearly that PLCβ1 is required for the agonist-induced endocannabinoid release and that the production of DAG, which is the precursor of 2-AG and detected by TRPC6, is closely correlated with the agonist-induced endocannabinoid release. These results support the hypothesis that 2-AG mediates the agonist-induced retrograde suppression.

It has been reported that depolarization-induced suppression of IPSC (DSI) was enhanced in the presence of DHPG or oxo-M (Ohno-Shosaku et al., 2002a, 2003; Varma et al., 2001). This apparent "DSI enhancement" can result either from potentiation of the DSI mechanism itself or from summation of DSI and the enhanced component of agonist-induced suppression of IPSC by depolarization. Our data clearly show that the latter is the case. We demonstrate that PLCβ1 is not required for DSI but is indispensable for both agonist-induced suppression of IPSC and its enhancement by depolarization. Thus, DSI and DSI enhancement involve distinct intracellular signaling cascades. At this stage, it is not known which of the two endocannabinoids, 2-AG or anandamide, mediates DSI. If 2-AG is involved, PLCδ isozymes might play an essential role because they can be activated by Ca^{2+} alone (Rebecchi and Pentyala, 2000; Rhee, 2001).

Agonist-Induced Suppression of IPSC and $[Ca^{2+}]_i$

Our data indicate that the agonist-induced suppression of IPSC is not evident at pCa 8–9. However, several previous studies reported that application of DHPG (Maejima et al., 2001a; Ohno-Shosaku et al., 2002a; Varma et al., 2001) or oxo-M (Fukudome et al., 2004) could induce endocannabinoid-mediated suppression of synaptic transmission even under nominally low $[Ca^{2+}]_i$ conditions. One possibility of this apparent discrepancy is that the agonist concentrations were different. In the previous studies, we mainly used 50 μM DHPG and 3 μM oxo-M, which were ten times higher than those used in the present study. Thus, it is possible that, even at a low $[Ca^{2+}]_i$ level, such a high dose of agonist activated PLCβ to the extent sufficient to produce 2-AG that could suppress synaptic transmission. Additionally, it is also possible that the $[Ca^{2+}]_i$ of the postsynaptic neuron might have been higher than the level expected from the composition of the pipette solution. In most experiments of our previous studies with hippocampal neurons, we had to induce DSI in the beginning to confirm that the IPSCs under investigation were cannabinoid sensitive. Such a treatment (5 s depolarization to 0 mV) can induce large Ca^{2+} transients, and it takes several minutes for the $[Ca^{2+}]_i$ to return to the original level. Therefore, in these experiments, the $[Ca^{2+}]_i$ might have been somewhat elevated at the time of agonist application, even though evoked IPSC amplitude recovered to the control level. In the present study, to minimize such a possible shift of basal $[Ca^{2+}]_i$, we applied 2-AG instead of depolarization to confirm the cannabinoid sensitivity of IPSCs.

Measurements of PLCβ Activity by TRPC6 Channels

PLC activity can be assayed directly by measuring the production of IP_3 or some other related molecules or indirectly by measuring $[Ca^{2+}]_i$ transients caused by IP_3-induced Ca^{2+} release. In addition to these two classical methods, two new techniques have been developed recently. One is the optical method using fluorescent biosensors targeting various components of the PLC signaling system, such as IP_3 and DAG (Hirose et al., 1999; Nahorski et al., 2003; Oancea et al., 1998). The other is the electrophysiological method using TRPC channels as biosensors for PLC products (Delmas et al., 2002). In the present study, we used the latter method because it enables us to detect real-time activity of PLCβ within individual intact cells while simultaneously controlling their membrane potential and $[Ca^{2+}]_i$.

The use of TRPC6 channel as a DAG sensor was originally developed in rat sympathetic neurons (Delmas et al., 2002). In the present study, we confirmed that TRPC6 was activated by DAG, but not by store depletion, and that this method is applicable to mammalian central neurons. We found, however, that special care should be taken to use this method. First, Ca^{2+} influx through TRPC6 channels disturbs the cell milieu. We obtained the data showing that TRPC6-mediated Ca^{2+} influx can induce a local $[Ca^{2+}]_i$ elevation, and additionally activate PLCβ even in the presence of 10 mM BAPTA (data not shown). In this study, we minimized this artifact by removing external Ca^{2+} or by reducing the duration of agonist application. Second, repetitive application of an agonist induces rundown of TRPC6 currents, mostly in an activity-dependent manner. To reduce rundown, the amplitude of each response should be minimized. Finally, endogenous inward currents induced by agonist application might disturb the signal derived from exogenous TRPC6 channels. There are several studies showing that the activation of mGluRs induces inward currents in hippocampal pyramidal cells (Crepel et al., 1994; Gee et al., 2003; Rae and Irving, 2004). Induction of such inward currents by DHPG or oxo-M was, however, observed only in a minor fraction of control neurons in our culture system (3 out of 20 neurons for oxo-M, 3 out of 19 neurons for DHPG). In the majority of control neurons, neither oxo-M nor DHPG generated detectable inward currents (Figures 5B and 5C). Thus, this method is useful for the measurement of PLC activity in cultured hippocampal neurons, when special care is taken.

$[Ca^{2+}]_i$ Dependence of PLCβ

The activity of PLCβ as well as other types of PLC is Ca^{2+} dependent (Rebecchi and Pentyala, 2000; Taylor et al., 1991). Considering physiological significance, whether PLCβ activity is saturated or submaximal for Ca^{2+} at physiological levels is very important. However, it is difficult to answer this question with biochemical methods, because the Ca^{2+} dependence of PLCβ activity assayed biochemically can be different under differ-

ent assay conditions, such as enzyme preparation (isolated or membrane bound) and supplement of various interactive factors including GTP and G proteins (del Rio et al., 1994; Fisher et al., 1989; Ryu et al., 1987; Smith et al., 1986; Taylor et al., 1991; Taylor and Exton, 1987; Uhing et al., 1986). In the present study, we estimated the Ca^{2+} dependence of PLCβ activation in intact neurons by using the bioassay technique with the TRPC6 channel, and we have demonstrated that the PLCβ activity is submaximal at resting $[Ca^{2+}]_i$ levels, mostly lower than 100 nM, and sensitive to a small change in $[Ca^{2+}]_i$. Our results strongly suggest that the synergism between Ca^{2+} elevation and receptor activation occurs at the level of PLCβ1 (see below). However, we cannot exclude a possibility that an additional Ca^{2+}-dependent element upstream from PLCβ could also be involved.

Several previous studies show that receptor-mediated Ca^{2+} mobilization is enhanced by increasing $[Ca^{2+}]_i$ (Irving and Collingridge, 1998; Masgrau et al., 2000; Nakamura et al., 1999; Wang et al., 2000). This enhancement has been explained either by a change in loading state of Ca^{2+} store (Irving and Collingridge, 1998; Masgrau et al., 2000) or by a Ca^{2+} sensitivity of IP_3R (Nakamura et al., 1999; Wang et al., 2000). Our present study suggests that, in addition to these two mechanisms, the enhancement of PLCβ activation itself by Ca^{2+} might also partly contribute to the enhancement of receptor-mediated Ca^{2+} mobilization, as suggested previously (Masgrau et al., 2001; Nakamura et al., 2002).

Physiological Significance of the Ca^{2+} Dependency of PLCβ

What is the physiological significance of the Ca^{2+}-dependent nature of PLCβ? First, it may contribute to the formation of a positive feedback loop between PLCβ activity and $[Ca^{2+}]_i$. Activation of PLCβ induces an elevation of $[Ca^{2+}]_i$, which in turn induces further activation of PLCβ if receptor ligands still exist. Through this feedback loop, the receptor-$G_{q/11}$-PLCβ signaling pathway can be driven in a regenerative manner. PLCβ-induced $[Ca^{2+}]_i$ elevation in this loop might be caused by IP_3R-mediated Ca^{2+} release. In certain cell types, activation of Ca^{2+}-permeable TRPC channels might also contribute to the $[Ca^{2+}]_i$ elevation. The efficiency of the positive feedback mechanism is maximized by the close apposition of PLCβ and IP_3R or TRPC channels. Recent studies have shown such a molecular organization of these signaling elements by scaffold and anchoring proteins (Delmas et al., 2004).

Second, the Ca^{2+}-dependent nature of PLCβ can contribute to coincidence detection, as demonstrated in the present study. When weak activation of $G_{q/11}$-coupled receptors, which by itself is not sufficient to activate PLCβ, coincides with postsynaptic depolarization, the PLCβ-dependent signaling pathway can be driven effectively. Thus, this pathway can sense the coincidence of the presynaptic (transmitter release) and postsynaptic (depolarization) activity through the Ca^{2+} dependency of PLCβ. PLCβ can be activated by various neurotransmitters or modulators through the corresponding receptors. It is therefore likely that PLCβ contributes to various types of activity-dependent synaptic modulations as a coincidence detector.

Experimental Procedures

Preparation of Neurons

Cultured hippocampal neurons were prepared from newborn rats or mice (*PLCβ1*$^{+/+}$ and *PLCβ1*$^{-/-}$) (Kim et al., 1997) as described previously (Ohno-Shosaku et al., 2001). The cultures were kept at 36°C in 5% CO_2 for 10–15 days before use.

Exogenous TRPC6 channels were expressed in cultured hippocampal neurons. The cDNA for mouse TRPC6 was kindly provided from Dr. Yasuo Mori (Kyoto University, Kyoto, Japan) and was subcloned into pCI-neo vector. A mixture of plasmid cDNAs for TRPC6 and EGFP (Clontech, 18–133 ng/μl) was loaded into a glass capillary (Femtotips II, Eppendorf) and pressure injected into the nucleus of hippocampal neurons with a programmable microinjector (FemtoJet, Eppendorf). The concentration of TRPC6 cDNA was 67 ng/μl for measurements of oxo-M- or DHPG-induced currents and 182 ng/μl for OAG-induced currents. The injected neurons were further incubated for 9–24 hr before electrophysiological experiments. As a control, we used the cells injected with the cDNA for EGFP and the empty vector. The transfected neurons were identified with the green fluorescence of EGFP.

Electrophysiology

The neurons were whole-cell clamped with patch pipettes, and the current responses were recorded at −80 mV with a patch-clamp amplifier (EPC9/3 or EPC10/2, HEKA). The amplitude of current responses was measured at the peak. For recording IPSCs, both presynaptic and postsynaptic neurons were whole-cell clamped. The presynaptic neuron was stimulated by applying voltage pulses (to 0 mV, 2 ms) at 0.2 Hz, and the evoked IPSCs were recorded from the postsynaptic neuron. The magnitude of agonist-induced suppression was calculated from mean amplitudes of 6 to 15 consecutive IPSCs in the presence and absence of the agonist. The magnitude of depolarization-induced suppression was calculated from mean amplitudes of 12 consecutive IPSCs before depolarization and seven consecutive IPSCs obtained 4–18 s after depolarization. All experiments were performed at room temperature (24°C–26°C).

Ca^{2+} Measurements

Neurons were loaded with a Ca^{2+} indicator dye (fura-2, 200 μM) through patch pipettes. Fluorescence signals for excitations of 340 nm (F340) and 380 nm (F380) were measured at 0.2 Hz at the soma and dendrites by means of a cooled-CCD camera system (Acquacosmos, Hamamatsu Photonics, Japan). To convert fluorescence ratios to $[Ca^{2+}]_i$, we performed an in vivo calibration with three different solutions (Almers and Neher, 1985), using the following formula: $[Ca^{2+}]_i = K_{eff}(R - R_{min})/(R_{max} - R)$, where R is the ratio value, R_{min} is the ratio for a Ca^{2+}-free solution (30 mM BAPTA), R_{max} is the ratio for a saturated Ca^{2+} solution (2 mM Ca^{2+}). K_{eff} was obtained by the following formula: $K_{eff} = 100(R_{max} - R_{pCa7})/(R_{pCa7} - R_{min})$ nM, where R_{pCa7} is the ratio for pCa 7 intracellular solution.

Solutions

A standard external solution contained 140 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, and 10 mM glucose (pH 7.3, adjusted with NaOH). For recording TRPC6 currents and IPSCs, the external solution was supplemented with 0.1 μM tetrodotoxin and 1 mM kynurenic acid, respectively. In some experiments, we used a Ca^{2+}-free external solution made by substituting Mg^{2+} for Ca^{2+}. The recording chamber was perfused with an external solution with or without drugs at a flow rate of 1–3 ml/min. For the induction of TRPC6 currents, an external solution containing a drug was locally applied through an ~250 μm wide capillary tube located near the neuron using a perfusion valve controller (VC-6M, Warner Instruments). To wash out the drug, the bath was continuously perfused with a drug-free solution. In a brief application protocol, we used a 1 ms command pulse. Under this condition, the actual exposure time was estimated to be 2–3 s by measuring the shift of holding current with a high K^+ (20 mM) solution.

Low doses of agonists (0.3 μM oxo-M and 5 μM DHPG) were used for measuring IPSCs, whereas high doses (3 μM oxo-M and 50 μM DHPG) were required for eliciting TRPC6-mediated currents.

This difference in agonist dose is because IPSCs are sensitive to a nanomolar range of endocannabinoids, whereas TRPC6 channels are sensitive to a micromolar range of DAG. If the high doses are used for IPSC measurements, IPSCs are fully suppressed even at pCa 7, making it difficult to examine the Ca^{2+} dependence between pCa 6 and 7. Conversely, the amplitudes of TRPC6 currents induced by the low doses of agonists were too small to do quantitative measurements.

For presynaptic neurons in IPSC measurements, we used an internal solution containing 120 mM K-gluconate, 15 mM KCl, 6 mM $MgCl_2$, 5 mM EGTA, 10 mM HEPES, 20 mM KOH, and 5 mM Na_2ATP (pH 7.3 adjusted with KOH). For measuring TRPC6 currents, or for postsynaptic neurons in IPSC measurements, we used internal solutions adjusted to pCa 9, pCa 8, pCa 7, and pCa 6. These solutions contained 113.4 mM K-gluconate, 15 mM KCl, 10 mM HEPES, 10 mM BAPTA, 5 mM $MgCl_2$, 5 mM Na_2ATP, 0.2 mM Na_2GTP, and appropriate concentrations of $CaCl_2$. The pCa was calculated using a computer program (WinMAXC v2.45 obtained at http://www.stanford.edu/~cpatton/maxc.html). The pH of each solution was adjusted to 7.3 with KOH and the osmolality was maintained at 290–300 mOsm with K-gluconate. In some experiments, we also used internal solutions containing 30 mM BAPTA or 10 mM EGTA instead of 10 mM BAPTA. The electrode resistance ranged from 2 to 5 MΩ when filled with an internal solution.

Slices

Hippocampal slices were prepared from 3- to 4-week-old inbred C57BL/6 mice, as described previously (Ohno-Shosaku et al., 2002b). The slice was superfused continuously with the normal solution containing 125 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 1.25 mM $NaHPO_4$, 26 mM $NaHCO_3$, and 10 mM glucose, bubbled with a mixture of 95% O_2 and 5% CO_2 (pH 7.4). Electrical recordings were made from CA1 pyramidal neurons in slices using patch pipettes (5–7 MΩ) at room temperature. The pipette solution contained 130 mM CsCl, 5 mM KCl, 5 mM NaCl, 10 mM HEPES, 0.6 mM EGTA, 4 mM MgATP, and 0.3 mM GTP, pH adjusted to 7.3 with NaOH. Whole-cell tight seals (>5 GΩ) were made on the soma, and the capacitance was fully compensated by patch-clamp amplifier (EPC-9, HEKA). The range of series resistance we accepted for recordings was 10–15 MΩ. The membrane potential of the neurons was held at −70 mV. Cells were identified as pyramidal neurons using both electrical and anatomical criteria. A couple of bipolar stimulation electrodes constructed from thin tungsten wire (50 μm OD) were placed on both the stratum radiatum and the stratum oriens in the CA2–3 regions. These stimulation electrodes were used to evoke IPSCs and to stimulate cholinergic inputs, respectively. A non-NMDA receptor antagonist (6-cyano-7-nitroquinoxaline-2,3-dione [CNQX], 10 μM), an NMDA receptor antagonist (DL-2-amino-5-phosphonovaleric acid [APV], 50 μM), a group I and II mGluR antagonist [(S)-a-methyl-4-carboxyphenylglycine (MCPG), 500 μM], a mGluR5-specific antagonist [2-methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP), 5 μM], and a choline esterase inhibitor (eserine, 10 μM) were always added to the perfusing solution. In some experiments, MCPG was replaced with an mGluR1 antagonist, 7-(hydroxyimino)cyclopropa(b)chromen-1a-carboxylate ethyl ester (CPCCOEt, 100 μM).

Statistics

Averaged data from different experiments are presented as mean ± SEM. Statistical significance was assessed by Student's t test. One, two, and three asterisks or sharps indicate $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

Chemicals

MCPG, MPEP, CPCCOEt, DHPG, oxo-M, and thapsigargin were purchased from Tocris Cookson (UK). U73122 was from Biomol, 2-AG was from Cayman, atropine was from Nacalai Tesque (Japan), and APV, CNPX, eserine and calmidazolium were from Sigma-Aldrich. A stock solution of OAG (Sigma) in DMSO was added to the solution containing albumin (0.2 mg/ml) and sonicated for 3–5 min before use for local perfusion. Since 2-AG is rapidly converted to 1-AG or 3-AG in an aqueous solution, a stock solution of 2-AG in DMSO was added to the external solution just before use.

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